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A soluble recombinant polypeptide comprising the amino-terminal half of the extracellular region of the CD4 molecule contains an active binding site for human immunodeficiency virus

(epitope mapping/envelope glycoprotein binding/acquired immunodeficiency syndrome/retrovirus receptor/vaccinia virus expression system)

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ABSTRACT **Infection of helper T lymphocytes by human immunodeficiency virus is initiated by a specific interaction of the viral envelope glycoprotein with CD4, an integral membrane glycoprotein of the target cell. We have adapted a vaccinia virus-based mammalian cell expression system to produce variants of the CD4 molecule for structure–function studies. In this report we demonstrate that a truncated 180-amino acid fragment representing approximately the N-terminal half of the extracellular region of CD4 is found primarily in soluble form in the extracellular medium. Epitope analysis with a panel of anti-CD4 murine monoclonal antibodies indicates that the fragment reacts with those antibodies known to block the interaction between CD4 and the human immunodeficiency virus envelope glycoprotein but reacts poorly or not at all with those antibodies that do not block this interaction. We also show that the fragment forms a specific complex with a soluble form of gp120, the CD4-binding subunit of the viral envelope glycoprotein. These results indicate that this soluble CD4 fragment contains an active binding site for human immunodeficiency virus.**

CD4 is an integral membrane glycoprotein of human helper T lymphocytes that serves as an essential component of the receptor for the human immunodeficiency virus (HIV) (1–6), the causative agent of acquired immunodeficiency syndrome. HIV binding and fusion with the cell are mediated by specific interaction between the external subunit of the viral envelope glycoprotein (gp120) and CD4 on the target cell surface (5, 7–13). Recent studies have yielded considerable insight into the structure of the CD4 molecule and its counterpart from other species. The primary sequence deduced from the human cDNA (14) indicates that the processed molecule is ≈435 amino acid residues in length, with a long N-terminal extracellular region followed by a transmembrane segment and a C-terminal cytoplasmic tail. The external region contains an N-terminal domain of ≈100 amino acid residues that shares striking sequence homology and secondary structural features with the immunoglobulin light chain variable domain. The remainder of the external region (≈270 residues) appears to be composed of three additional domains that also display structural relationships to the immunoglobulin family (15). The finding of introns separating the coding sequences for these regions in the CD4 gene (16) supports this notion of structural and possibly functional domains. Of particular interest is the existence of conserved pairs of cysteine residues that probably form intradomain disulfide bonds within the first, second, and fourth external domains (17). These structural features deduced from cDNA sequencing have been complemented by epitope analyses using panels of anti-CD4 monoclonal antibodies (mAbs). Such studies have shed light on the topographic relationships of the various epitopes to one another, to the HIV-binding site, and to the cell membrane (13, 18–21). However, the region(s) of the CD4 molecule involved in binding to the HIV envelope glycoprotein have yet to be identified.

Our approach to this problem involves testing variants of the CD4 molecule (produced by recombinant DNA technology) for properties associated with the HIV-binding site. In this report we describe a soluble truncated fragment of ≈180 amino acid residues, representing approximately the N-terminal half of the extracellular region of CD4, which has immunological and functional properties indicative of the presence of an active HIV-binding site.

MATERIALS AND METHODS

Enzymes. Restriction endonucleases were obtained from New England Biolabs or Bethesda Research Laboratories. The Klenow fragment of DNA polymerase I and T4 DNA ligase were from New England Biolabs.

Antibodies. Murine anti-CD4 mAbs were obtained from the following sources: MT151, Boehringer Mannheim; Leu3A, Becton Dickinson; OKT4, OKT4A, OKT4B, OKT4C, OKT4D, OKT4E, and OKT4F, M. Talle, Ortho Diagnostics. Two murine anti-gp120 mAbs were employed: 2E12.1 (Epitope, Beaverton, OR) and a tissue culture supernatant from hybridoma 902 (B. Chesebro, National Institute of Allergy and Infectious Diseases, Hamilton, MT). Rabbit antiserum to mouse IgG (heavy plus light chains) was purchased from ICN.

Plasmids. The CD4 cDNA was donated by D. Littman (University of California, San Francisco). Plasmid pCD4-GEM4 (obtained from A. Rabson, National Institute of Allergy and Infectious Diseases, Bethesda, MD) contains a full-length copy of the CD4 cDNA with 5′ *Eco*RI and 3′ *Bam*HI linkers (14) cloned into the *Eco*RI–*Bam*HI site of pGEM4 (Promega Biotec, Madison, WI). pTF7-5 contains a bacteriophage T7 promoter and terminator separated by a unique *Bam*HI site and flanked by the left and right vaccinia thymidine kinase gene sequences (see Fig. 1).

Construction and preparation of recombinant plasmids were performed according to the methods outlined by Maniatis *et al.* (22). DNA fragments were purified from low-melting-point agarose gels by using the Elutip-d procedure (Schleicher & Schuell). Plasmids were isolated by the alkaline $NaDodSO_4$ lysis method (23) and purified by CsCl/ethidium bromide equilibrium density gradient centrifugation.

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Abbreviations: HIV, human immunodeficiency virus; mAb, monoclonal antibody; pfu, plaque-forming units.

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Virus and Cells. Vaccinia virus recombinant vTF7-3 contains the bacteriophage T7 gene 1 (encoding the T7 RNA polymerase) under control of the vaccinia P7.5 promoter (24). vPE6 is a vaccinia recombinant derived from pTF7-5 containing a bacteriophage T7 promoter linked to the HIV-1 envelope gene (IIIB isolate, clone BH8) with a termination codon inserted by *in vitro* mutagenesis immediately preceding the sequence encoding the consensus retroviral envelope cleavage site Arg-Glu-Lys-Arg (P. Earl, National Institute of Allergy and Infectious Diseases, Bethesda, MD, personal communication). This virus directs high-level expression of a secreted form of gp120 in cells doubly infected with vTF7-3.

CV-1 monkey kidney cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum.

Expression Conditions. Transfection experiments for transient expression were performed by using conditions similar to those described (24). CV-1 cells were grown to 90–95% confluence in 25-cm^2 flasks ($\approx 2.5 \times 10^6$ cells) and infected with vTF7-3 at a multiplicity of 30 plaque-forming units (pfu) per cell in medium with 2.5% fetal bovine serum. The virus was allowed to adsorb for 30 min at 37°C with occasional rocking of the flask, whereupon the inoculum was removed and replaced with 1 ml of transfection buffer containing 5 μg of calcium phosphate-precipitated plasmid DNA. After incubation for 30 min at 37°C with occasional rocking, 5 ml of medium containing 2.5% fetal bovine serum was added. The medium was removed after 4 hr at 37°C, and the cells were incubated with 2–4 ml of cysteine-free medium containing 2.5% dialyzed fetal bovine serum for 15–60 min. This medium was then replaced with 2.25 ml of the same medium supplemented with 0.1–0.3 mCi of L-[^{35}S]cysteine per ml (Amersham, 1.3 Ci/mmol; 1 Ci = 37 GBq). Labeling was allowed to proceed at 37°C for 4–5 hr, after which 0.25 ml of complete medium containing 2.5% fetal bovine serum was added. Thirty-six hours later the medium was collected and centrifuged in a Savant high-speed microcentrifuge, first at 2000 rpm for 5 min and then at 10,000 rpm for 30 min. The resulting supernatant was used for subsequent analyses.

For expression of secreted gp120, double virus infections were performed by using protocols similar to those reported (25). CV-1 cells grown in flasks as described above were infected with vTF7-3 and vPE6 (15 pfu each per cell) in 1 ml of medium containing 2.5 % fetal bovine serum. After 90 min at 37°C, the virus inoculum was removed and replaced with fresh medium containing 2.5% fetal bovine serum. In the case of unlabeled infection, the incubation was continued at 37°C for 23 hr, after which the medium was collected. For metabolic labeling, the incubation was continued for 10.5 hr, at which time the medium was removed and the cells were incubated for 15 min at room temperature in cysteine-free medium with 2.5% dialyzed fetal bovine serum. This medium was then replaced with 2.25 ml of the same medium supplemented with 0.1 mCi of [^{35}S]cysteine per ml. After 5 hr of labeling at 37°C, 0.25 ml of complete medium containing 2.5% fetal bovine serum was added. The incubation was continued for an additional 7 hr and the medium was then collected. Media from these infections were centrifuged as described above for the transfection experiments.

Control infections were performed identically, except that the virus inoculum contained vTF7-3 only (30 pfu per cell).

Radioimmunoprecipitation. The specific reaction conditions are described for each experiment in the figure legends. Protease inhibitor buffer contained 0.1 mM N^{α}-(*p*-tosyl)-lysine chloromethyl ketone, 0.1 mM L-1-tosylamido-2-phenylethyl chloromethyl ketone, 50 mM iodoacetimide, 0.01 mM leupeptin, and 70 Kallikrein units of aprotinin per ml in phosphate-buffered saline with 0.02% (wt/vol) sodium azide. Immune complexes collected with protein A-agarose (Calbiochem) were precipitated and washed three times by centrifugation in a Savant high-speed microcentrifuge at 3000 rpm for 5 min.

NaDodSO$_4$/Polyacrylamide Gel Electrophoresis. The procedure of Laemmli (26) was employed, with the acrylamide concentrations specified in the figure legends. Gels were analyzed by fluorography using EN^{3}HANCE (New England Nuclear). ^{14}C-Methylated protein molecular weight markers (Amersham) were lysozyme (M_r 14,000), carbonic anhydrase (M_r 30,000), ovalbumin (M_r 46,000), bovine serum albumin (M_r 69,000), phosphorylase b (M_r 93,000), and myosin (M_r 200,000).

RESULTS

Expression of the Soluble CD4 Fragment. The expression system employed in the present study is based on that described by Fuerst *et al.* (24). Mammalian cells are infected with a recombinant vaccinia virus (vTF7-3) containing the bacteriophage T7 RNA polymerase gene linked to a vaccinia promoter and then transfected with a plasmid vector containing the target gene of interest flanked by bacteriophage T7 promoter and transcriptional terminator regions. The T7 RNA polymerase mediates high-level transcription of the target gene in the cytoplasm of the transfected cells. We designed a plasmid vector (pEB-2) that contains, between the T7 promoter and transcriptional terminator, two new unique restriction sites (*Eco*RI and *Stu* I) directly followed by a universal translational termination sequence. After cleaving this vector with *Eco*RI and *Stu* I, any DNA fragment containing a 5′ *Eco*RI site and a 3′ blunt end can be force-cloned in the proper orientation. If the DNA insert contains the translation initiation codon but only a portion of the adjacent coding region for a particular gene, a truncated polypeptide will be expressed. Depending on which synthetic termination codon is in frame, the shortened polypeptide may also contain up to three additional C-terminal amino acids encoded by the vector.

For the present studies, we inserted the *Eco*RI–*Nhe* I DNA fragment of the CD4 cDNA into pEB-2 to obtain another plasmid, designated pCD4$_f$ (Fig. 1). Based on the reported cDNA sequence (14), pCD4$_f$ would be expected to encode a truncated variant of CD4 with a normal N terminus; cleavage of the presumed signal sequence would result in a polypeptide of about 180 amino acid residues representing approximately half of the extracellular region of CD4 and containing no consensus N-linked glycosylation sites. This fragment might therefore be expected to be secreted into the medium. Fig. 2 shows the results of a transient metabolic labeling experiment with cells infected with vTF7-3 and transfected with different plasmids. NaDodSO$_4$/polyacrylamide gel electrophoretic analysis revealed that the medium of cells transfected with control plasmid pEB-2 contained a complex pattern of polypeptide bands (lane 3); medium from cells transfected with pCD4$_f$ contained the same complex pattern of polypeptides as well as an additional faint band at the position expected for the truncated CD4 polypeptide encoded by this plasmid (lane 1). Immunoprecipitation analysis with a mixture of several murine anti-CD4 mAbs confirmed that this band indeed represented a fragment of the CD4 molecule: it was selectively removed from the medium after immunoprecipitation (lane 2) and was specifically precipitated from the medium of cells transfected with pCD4$_f$ (lane 5) but not pEB-2 (lane 6). The amounts of this labeled polypeptide that could be immunoprecipitated from the medium fraction far exceeded the amounts precipitable from the detergent-solubilized cell pellet fraction (data not shown). We conclude that pCD4$_f$ encodes the expected fragment representing the N-terminal half of the extracellular region of the CD4 molecule, that this fragment is secreted

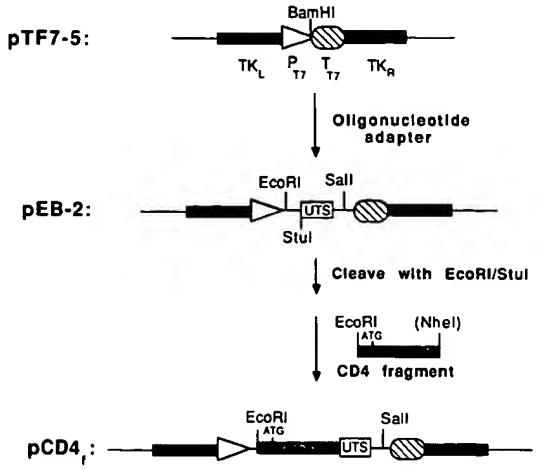

FIG. 1. Construction of plasmid $pCD4_f$. Plasmid pTK7-5 contains the bacteriophage T7 gene 10 promoter (P_{T7}) and the T7 terminator (T_{T7}) separated by a unique *Bam*HI site. This region is flanked by vaccinia virus thymidine kinase left (TK_L) and right (TK_R) sequences. An adapter made by using two partially complementary synthetic oligonucleotides (GATCGAATTCAGGCCTAATTAATTAAGTCGAC and GATCGTCGACTTAATTAATTAGGCCTGAATTC) was ligated into the *Bam*HI site of pTF7-5 by using the *Bam*HI 5′ overhangs of the adapter. The *Bam*HI site is destroyed in the desired recombinants. Hence the reaction mixture was digested with *Bam*HI to linearize recircularized plasmids lacking the insert, and ampicillin-resistant transformants were screened by restriction mapping to identify those containing the insert in the desired orientation. Plasmid pEB-2 contains unique sites for *Eco*RI and *Stu* I, followed by a universal termination sequence (UTS) providing termination codons in all three reading frames, followed by a unique *Sal* I site. The desired CD4 DNA fragment was obtained by digesting pCD4-GEM4 with *Nhe* I (which cleaves the plasmid at a unique site at nucleotide 678 of the CD4 cDNA sequence), filling in the staggered end with the Klenow fragment of DNA polymerase I and dNTPs, and then digesting with *Eco*RI. The resulting 0.68-kilobase *Eco*RI–*Nhe* I fragment, which contains the ATG initiation codon of CD4, was force-cloned into pEB-2, which had been digested with *Eco*RI and *Stu* I. The *Stu* I site in the vector is destroyed in the recombinant. Hence the reaction mixture was digested with *Stu* I, and $pCD4_f$ was identified by restriction mapping of the ampicillin-resistant transformants.

in soluble form into the medium, and that it displays reactivity with anti-CD4 mAbs.

Epitope Analysis. By using a variety of assays, several laboratories have demonstrated that specific anti-CD4 mAbs differ widely in their ability to block the interaction between CD4 and the HIV envelope glycoprotein (5, 7, 12, 13, 20, 21). It was therefore of interest to test which epitopes are expressed on the soluble CD4 fragment encoded by $pCD4_f$. Fig. 3 shows the results obtained by radioimmunoprecipitation analysis from the medium of cells metabolically labeled after transfection with $pCD4_f$. Interestingly, we observed a close correlation between the reactivity of a particular mAb with the fragment and the reported ability of the mAb to block the CD4–envelope glycoprotein interaction. Thus, strong immunoprecipitation was obtained with mAbs MT151, Leu3A, OKT4A, OKT4B, OKT4D, OKT4E, and OKT4F, all of which have been shown to inhibit the interaction of membrane-associated (5, 7, 20, 21) or soluble (12, 13) gp120 with CD4. By contrast, mAb OKT4 failed to immunoprecipitate the fragment, consistent with its reported inability to block CD4 interaction with membrane-associated (5, 7, 20, 21) or soluble (12, 13) gp120. mAb OKT4C displayed barely detectable reactivity with the fragment, in keeping with its reported failure to block interaction of intact HIV with CD4 (20, 21) and its relatively weak capacity to inhibit binding of soluble gp120 to CD4 (13). Several of the

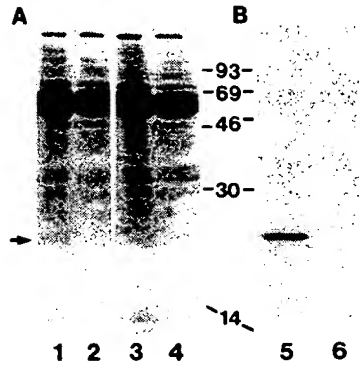

FIG. 2. Analysis of the metabolically labeled transient expression products. Transient metabolic labeling reactions were performed in cells infected with vTF7-3 and transfected with either plasmid pEB-2 or $pCD4_f$, and the media were collected. For immunoprecipitation, reaction mixes were prepared containing 1.1 ml of transfection medium, 0.99 ml of protease inhibitor buffer, and 0.11 ml of 20% (vol/vol) Nonidet P-40. The samples were cleared by incubation with 0.13 ml of a 20% (vol/vol) suspension of protein A-agarose for 1 hr at 4°C; this was followed by centrifugation. To the supernatants were added 2 μg of each of the following murine mAbs: MT151, OKT4, and OKT4A (all are IgG2b). After overnight incubation, 0.05 ml of 20% suspension of protein A-agarose was added, and the incubations were continued for 3 hr at 4°C on a rotator. The samples were centrifuged and pellet and supernatant fractions were saved. The pellets were washed and treated with 0.1 ml of sample buffer containing 8 M urea, and 0.09-ml aliquots were analyzed by gel electrophoresis. (*A*) Analysis on 12% gels of the total medium fractions (0.05 ml) and the corresponding supernatant fractions remaining after radioimmunoprecipitation. Lanes 1 and 2, total medium of cells transfected with $pCD4_f$ (lane 1) and the corresponding immunoprecipitation supernatant (lane 2); lanes 3 and 4, total medium of cells transfected with pEB-2 (lane 3) and the corresponding immunoprecipitation supernatant (lane 4). (*B*) Analysis on 15% gels of immunoprecipitates from medium of cells transfected with $pCD4_f$ (lane 5) and cells transfected with pEB-2 (lane 6). The arrow on the left indicates the position of the CD4 fragment band, and the numbers in the center represent molecular weight markers (expressed as $M_r \times 10^{-3}$).

anti-CD4 mAbs were also tested in an alternative assay that measures the ability of unlabeled medium from transfected cells to block the binding of a particular antibody to CD4 on the surface of a lymphocyte cell line. The results supported the immunoprecipitation analysis: medium from cells transfected with $pCD4_f$ blocked the binding of mAbs MT151, Leu3A, and OKT4A but had no effect on the binding of mAb OKT4 (data not shown). We conclude that the epitopes detected by the HIV-blocking anti-CD4 mAbs are contained within the N-terminal half of the extracellular region of CD4 and that functional epitopes for these mAbs can be produced when less than half of the full-length CD4 molecule is synthesized.

Interaction of the CD4 Fragment with Soluble gp120. We next sought to determine whether the CD4 fragment is capable of specific interaction with gp120. To test for the formation of a complex between these molecules, we mixed medium from metabolically labeled cells expressing soluble gp120 with medium from metabolically labeled cells expressing the CD4 fragment; we then assessed whether a murine anti-gp120 mAb could specifically coprecipitate the CD4 fragment along with the gp120. The results of such a coprecipitation experiment are shown in Fig. 4*A*. Of the many labeled bands observed in the initial mixture of media containing gp120 and the CD4 fragment (lane 1), the anti-gp120 mAb specifically precipitated two proteins: gp120 and the CD4 fragment (lane 6). The identity of the CD4 fragment band was confirmed by its absence when the fragment-containing medium was omitted from the reaction (only the

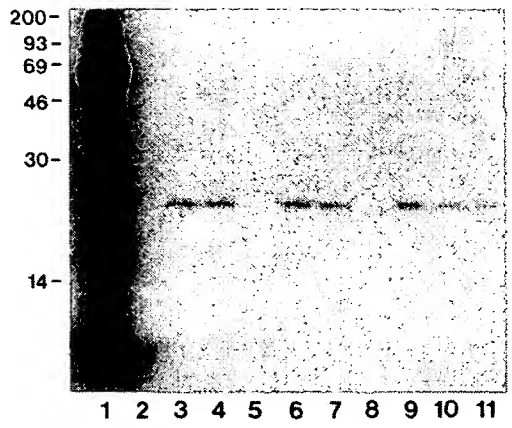

FIG. 3. Epitope analysis of the CD4 fragment. See text for preparation of medium from cells metabolically labeled after transfection with vTF7-3 and $pCD4_t$. A mixture was prepared containing 0.55 ml of this transfection medium, 0.55 ml of protease inhibitor buffer, 0.07 ml of 20% (vol/vol) Nonidet P-40, and 1.58 ml of phosphate-buffered saline containing 0.02% (wt/vol) sodium azide. The mixture was cleared with 0.28 ml of a 20% suspension of protein A-agarose as described in the legend to Fig. 2, and 0.27-ml aliquots (representing 0.05 ml of initial transfection medium) were treated with 1 μg of the indicated mAbs. After overnight incubation at 4°C, each sample received 0.05 ml of a 20% (vol/vol) suspension of protein A-agarose that had been previously coated with saturating amounts of rabbit antiserum to mouse IgG, thereby circumventing potential problems associated with antibodies of different subclasses. Samples were incubated on a rotator for 4 hr at 4°C, and the pellets were collected and washed. They were dissolved in 0.09 ml of sample buffer containing 8 M urea and applied to 12% polyacrylamide gels. Lane 1, total transfection medium (0.05 ml); lane 2, immunoprecipitate obtained with a control mAb (2E12.1). Immunoprecipitates were obtained with a battery of anti-CD4 mAbs: lane 3, MT151; lane 4, Leu3A; lane 5, OKT4; lane 6, OKT4A; lane 7, OKT4B; lane 8, OKT4C; lane 9, OKT4D; lane 10, OKT4E; lane 11, OKT4F. Molecular weight markers are shown on the left (expressed as $M_r \times 10^{-3}$).

gp120 band was observed, lane 3) and by its comigration with the single band observed when the fragment-containing medium was immunoprecipitated with an anti-CD4 mAb (lane 7). The presence of the CD4 fragment in the anti-gp120 immunoprecipitate resulted from a true complex with gp120, as judged by the absence of the fragment band when either normal medium (lane 4) or medium from metabolically labeled cells infected only with the vaccinia virus expressing T7 RNA polymerase (lane 5) was used in place of the gp120-containing medium. Analysis of the supernatants remaining after immunoprecipitation indicated that under the conditions of this experiment, nearly all of the CD4 fragment was complexed to gp120, and immunoprecipitation of the complex by the anti-gp120 mAb was virtually complete (data not shown). This suggests a reasonably high affinity for the binding reaction.

To further analyze the specificity of this interaction, we tested whether soluble unlabeled gp120 could compete for the immunoprecipitation of the CD4 fragment by an anti-CD4 mAb. As shown in Fig. 4*B*, unlabeled medium containing gp120 strongly inhibited the immunoprecipitation of the CD4 fragment by the OKT4A mAb (compare lanes 1 and 3). By contrast, medium lacking gp120 (from cells infected only with the vaccinia expressing T7 RNA polymerase) had no effect (lane 2). These results are consistent with previous reports that demonstrated HIV competition for binding of HIV-blocking mAbs to cell-associated CD4 (1, 5, 27). We conclude that the specificity of the interaction of the CD4 fragment with gp120 mirrors that previously described for

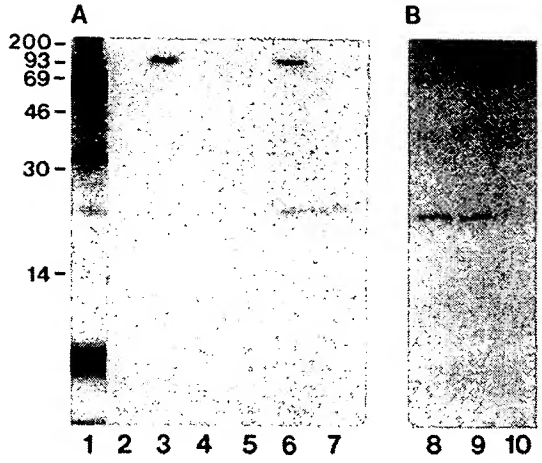

FIG. 4. Interaction of the CD4 fragment with gp120. Medium from cells metabolically labeled after infection with vTF7-3 and transfection with $pCD4_t$ was used as the source of the CD4 fragment. Media from unlabeled or metabolically labeled cells doubly infected with vTF7-3 plus vPE-6 served as the source of the gp120. Molecular weight markers are shown on the left (expressed as $M_r \times 10^{-3}$). (*A*) Coprecipitation of the CD4 fragment and gp120 by using an anti-gp120 mAb. Initial reaction mixtures contained 0.075 ml of each of the indicated media, 0.05 ml of protease inhibitor buffer, and 0.01 ml of 20% (vol/vol) Nonidet P-40. In the case of the double and single virus infection media, mixtures containing 1 part labeled and 19 parts corresponding unlabeled media were used. After preincubation for 4 hr at room temperature, 0.14-ml aliquots were removed and the indicated mAbs were added (anti-gp120, 0.1 ml of hybridoma 902 supernatant; anti-CD4, 1 μg of OKT4A). The immune complexes were collected with protein A-agarose that had been precoated with rabbit antiserum to mouse IgG and processed for electrophoresis on 12% gels as described in the legend to Fig. 3. Lane 1, total reaction mixture from the incubation containing the transfection medium and the vTF7-3 plus vPE-6 double infection medium. The immune precipitates were obtained from reactions containing the following additions: lane 2, transfection medium, vTF7-3 plus vPE-6 double infection medium, no antibody; lane 3, normal medium containing 2.5% fetal bovine serum, vTF7-3 plus vPE-6 double infection medium, anti-gp120; lane 4, transfection medium, normal medium containing 2.5% fetal bovine serum, anti-gp120; lane 5, transfection medium, vTF7-3 single infection medium, anti-gp120; lane 6, transfection medium, vTF7-3 plus vPE-6 double infection medium, anti-gp120; lane 7, transfection medium, normal medium containing 2.5% fetal bovine serum, anti-CD4. (*B*) gp120 inhibition of immunoprecipitation of the CD4 fragment by an anti-CD4 mAb. Initial reaction mixtures contained 0.05 ml of metabolically labeled transfection medium, 0.05 ml of protease inhibitor buffer, 0.01 ml of 20% Nonidet P-40, and 0.04 ml of phosphate-buffered saline containing 0.02% (wt/vol) sodium azide. The mixtures were supplemented with the unlabeled media indicated below and allowed to incubate at room temperature for 5 hr. Ten nanograms of anti-CD4 mAb OKT4A was added, and the incubations were continued overnight at 4°C. Immune complexes were collected and processed as described in the legend to Fig. 2 and electrophoresed on 12% gels. The supplementary media added during the initial incubation were as follows: lane 8, normal medium containing 2.5% fetal bovine serum; lane 9, vTF7-3 single infection medium; lane 10, vTF7-3 plus vPE-6 double infection medium.

the interaction of intact CD4 with the HIV envelope glycoprotein.

DISCUSSION

The results presented here provide direct biochemical evidence for localization of the HIV-binding site within the N-terminal half of the CD4 molecule. A recombinant fragment comprising this region contains a functional binding site for gp120 and reacts with all seven of the HIV-blocking mAbs

tested (MT151, Leu3A, OKT4A, OKT4B, OKT4D, OKT4E, and OKT4F). Previous mapping studies with these and other anti-CD4 mAbs have revealed the existence of multiple clusters of epitopes for mAbs that block the CD4–HIV envelope interaction (13, 18–21). It has been argued from trypsin digestion experiments with cell-associated CD4 (18) that these epitopes probably reside relatively distal to the cell membrane (18–20), an interpretation that is proven by our findings. We also found correlations with anti-CD4 mAbs that do not block HIV. The OKT4C mAb binds the fragment poorly, consistent with its nondetectable (20, 21) or weak (13) blocking activity for the CD4–HIV envelope interaction. The complete failure of the OKT4 mAb to react with the fragment suggests that the corresponding epitope resides within the membrane-proximal half of the external region of CD4, consistent with previous interpretations of the trypsin digestion experiments noted above (18–20). However, the possibility remains that this epitope is located within the N-terminal half of the external region but is not detected in the fragment due to conformational requirements. In any case, the failure of mAb OKT4 to react with the fragment is consistent with the finding that expression of this epitope is not required for HIV infectivity (28). Another point of note is that the fragment displays gp120 binding activity despite the absence of the two potential N-linked glycosylation sites present in the full-length sequence, both of which are located in the membrane-proximal half of the external domain (14). This carbohydrate independence of CD4 contrasts with the reported requirement of carbohydrate on gp120 for functional binding activity (27). However, our data do not exclude the possibility that regions in the C-terminal half of the external region of CD4 (including N-linked oligosaccharide chains) may influence HIV binding and other physiologically important functions in ways not measured by the assays employed here.

The present study localizes the HIV-binding site to a region that includes the first two structural domains of CD4. It will be most important to define more precisely the determinants directly involved in interaction with gp120. Further investigation is necessary to examine other soluble CD4 fragments and mutants and to prepare the corresponding vaccinia virus recombinants that will permit purification of large quantities of these polypeptides. In addition to enabling detailed biochemical studies of the CD4–gp120 interaction, such fragments will be analyzed for their ability to block HIV infectivity and for their effects on normal immunological functions believed to involve CD4 (16). The results of such studies will have important implications for the design of derivatives of the CD4 molecule with therapeutic potential in the treatment of acquired immunodeficiency syndrome.

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